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ARTERY WALL CALCIFICATION: CORRELATION OF ATHEROSCLEROSIS WITH MINERALIZATION

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Calcificazione della parete arteriosa: correlazioni tra l'aterosclerosi e la mineralizzazione. *Sono state compiute ricerche sull'arteria poplitea di 14 soggetti tra i 17 e 85 anni. Le concentrazioni e localizzazioni di P, S, Cl, K, Ca, Fe, Cu, Zn e Br sono state misurate con i metodi PIXE e micro-PIXE. La presenza dei gruppi PO_4^{3-} e CO_3^{2-} è stata accertata con la tecnica IR. La quantità di P e Ca aumenta con l'età circa del 9% e del 20%, e depositi minerali si trovano nella tunica media. Contemporaneamente è stato osservato un aumento nel rapporto Ca/P e nella cristallinità dei depositi. I campioni prelevati da soggetti anziani contengono pure gruppi CO_3^{2-}. Le concentrazioni e localizzazioni di Zn e Br mostrano cambiamenti dipendenti dallo strato della parete arteriosa. In alcuni punti della parete arteriosa si trovano minerali anche in soggetti giovani, senza correlazione con le zone delle ramificazioni dei vasi.*

Key words: *Popliteal artery - mineralization - atherosclerosis - proton microprobe.*

INTRODUCTION

Atherosclerosis is a complex process affecting a number of arteries. It is the principal cause of myocardial and cerebral infarctions that account for the majority of deaths in Western industrial societies. According to generally accepted criteria (1-3) four main stages can be recognized in the course of atherosclerotic process: normal arterial wall, fatty streak, fibrous plaque and so-called complicated lesion or advanced atherosclerotic lesion.

The most distinct characteristics of the advanced atherosclerotic lesions is the presence of mineral deposits. In agreement with the current concept (1-2), the formation of atherosclerotic lesions will not progress futher if the initial sequence does not occur, hence the formation of calcium deposits takes phase in the last phase of atherogenesis.

Although calcification has been considered as an indicator of the late stage of the disease, recent studies have demonstrated the importance of calcium in the onset and development of atherosclerosis (4). *Kramsch* et al. (5) found that rabbits fed an inorganic calcium antagonist ($LaCl_3$) and cholesterol did not develop lesions to the same extent as those fed cholesterol alone. Similar results have been reported for cholesterol-fed rabbits which additionally received intraperitoneal injections of calcium blocker nifidipine (6) and diltiazem (7).

These studies indicate that calcium ions are the crucial factor in the development of atherosclerosis and suggest that mineralization is not merely a complication of the disease, but that calcium within the artery wall may play an active role in its development.

It is the aim of this paper to study whether the formation of calcium-phosphate deposits (mineralization) can occur at the first stage of atherogenesis, i.e. in the artery wall without any morphological changes.

MATERIALS AND METHODS

Samples of human popliteal artery were collected from 14 patients (adults ranging in age from 17 to 85 y). All samples were obtained upon autopsy except 4 which were collected during vascular surgery (Table I no. 1, 2, 3 and 8).

The artery samples were always excised from the same region (the upper segment of the popliteal artery), immediately frozen in liquid nitrogen and divided into three parts.

One part was freeze-dried and crushed in an agate mill. The powder was used for the determination of the elemental composition of artery wall homogenates. As analytical method the proton induced X-ray emission (PIXE) technique was applied. The concentrations of P, S, Cl, K, Ca, Fe, Cu, Zn and Br were measured. The details of X-ray analysis are reported elsewhere (8, 9).

The second part of artery sample was cryo-sectioned at —25°C to 8 μm thick sections, placed on Formvar backing, dried and then subjected to irradiation with a proton microprobe to investigate the distribution of the trace element concentrations within the artery wall. For these measurements we used the proton microprobe (10) of the 3.75 MV Van de Graaff accelerator of the Karlsruhe Nuclear Research Center, with a minimal beam cross section of 3x3 μm^2. It enables to measure the elemental composition at the cellular level by the PIXE method. Simultaneously with the X-rays the backscattered protons were detected and used for the sample thickness determination. The results were obtained in the line-scan mode, each scanning line consisting of 256 equidistant points. In each irradiated point the section thickness as well as the concentrations of P, S, Cl, K, Ca, Fe, Cu, Zn and Br were measured. For morphological examination the sections were stained after irradiation with Mallory-azan method.

The third part of the material was calcined for 8 hours at 450°C. The mass of sample was recorded before and after incineration and infrared (IR) spectra were obtained using the SPECORD 75 IR (Carl Zeiss Jena) IR spectrometer.

RESULTS AND DISCUSSION

Every experimental method applied in this study (PIXE, micro-PIXE, IR spectroscopy) provides information from different levels of the morphological organization of the sample.

The elemental composition of popliteal artery homogenates are presented in Table I. The age of patients and the mass of ash after incineration expressed as % of dry mass are also presented in that table. The mass of ash reflects the amount of minerals and correlates with the age of patients (correlation coefficient 0.88) as reported previously (12). Since mineral deposits in a pathologically altered artery wall contain mainly calcium salts, most of which are phosphates, correlations of Ca and P concentrations with age were also found (correlation coefficients 0.92 and 0.77 respectively). The concentrations of other elements de-

TABLE I

Concentrations of P, S, Cl, K and Ca are expressed in mg/g of dry mass. For Fe, Cu, Zn and Br concentrations are expressed in μg/g of dry mass. Errors given for concentration values are standard deviations due to counting statistics.

Sample	*Age (Y)*	*Mass of ash (%)*	*P*	*S*	*Cl*	*K*	*Ca*	*Fe*	*Cu*	*Zn*	*Br*
1	17	0.90	4.3	8.9	12.2	7.5	0.9	250	4.4	183	97
+/—			0.4	0.7	1.0	0.6	0.2	31	1.0	28	19
2	21	0.83	2.9	7.1	7.9	4.9	1.3	223	223	732	85
+/—			0.3	0.6	0.6	0.5	0.3	27	19	42	17
3	23	0.62	4.4	11.7	8.9	5.8	2.7	427	321	836	141
+/—			0.4	0.9	0.7	0.5	0.3	36	25	49	22
4	49	0.94	6.0	12.8	9.4	7.3	3.3	216	4.1	199	54
+/—			0.5	1.0	0.8	0.6	0.3	24	0.9	32	9
5	54	2.6	4.6	12.1	8.5	6.3	3.2	166	9.4	77	103
+/—			0.4	1.0	0.7	0.5	0.2	19	2.3	13	15
6	63	5.1	5.9	10.0	7.4	4.8	4.4	110	3.6	92	71
+/—			0.5	0.9	0.6	0.4	0.3	16	1.1	12	11
7	67	3.8	6.1	11.4	7.3	3.7	4.6	192	11.1	117	114
+/—			0.5	0.9	0.6	0.3	0.3	27	3.2	20	18
8	70	4.5	6.8	9.0	10.0	4.0	4.1	140	18.2	122	109
+/—			0.6	0.7	0.9	0.3	0.3	18	5.3	17	15
9	74	3.4	5.7	7.2	8.5	4.1	4.2	275	4.5	182	61
+/—			0.4	0.5	0.7	0.3	0.3	32	1.3	24	8
10	78	6.7	5.0	7.3	8.1	5.5	5.1	172	5.7	72	44
+/—			0.4	0.6	0.6	0.4	0.3	20	1.7	11	5
11	79	8.4	5.3	8.5	9.8	6.2	4.4	140	6.9	133	82
+/—			0.4	0.6	0.7	0.5	0.4	19	2.1	21	12
12	83	9.0	7.0	7.5	8.3	4.7	6.0	95	8.8	159	61
+/—			0.5	0.5	0.6	0.4	0.5	14	3.7	23	8
13	85	7.2	5.8	9.0	10.2	4.9	6.6	144	7.4	59	94
+/—			0.4	0.7	0.9	0.4	0.5	21	2.7	11	10
14	85	7.6	7.0	8.1	9.0	3.5	5.1	307	10.3	148	73
+/—			0.5	0.6	0.7	0.3	0.4	36	3.9	22	10

termined in this study did not demonstrate correlations with age (e.g. for S, correlation coefficient amounts to —0.02). The mean concentration values for all measured elements are similar to those reported in ref. 13.

The use of the proton microprobe enabled the determination of both element content and distribution within the artery wall. It should be pointed out that this technique allows the examination in situ. An example of micro-PIXE results obtained for sample no. 1 is shown in Fig. 1. Fig. 2 illustrates the results for sample no. 2. In all figures tunica adventitia, tunica media as well as tunica

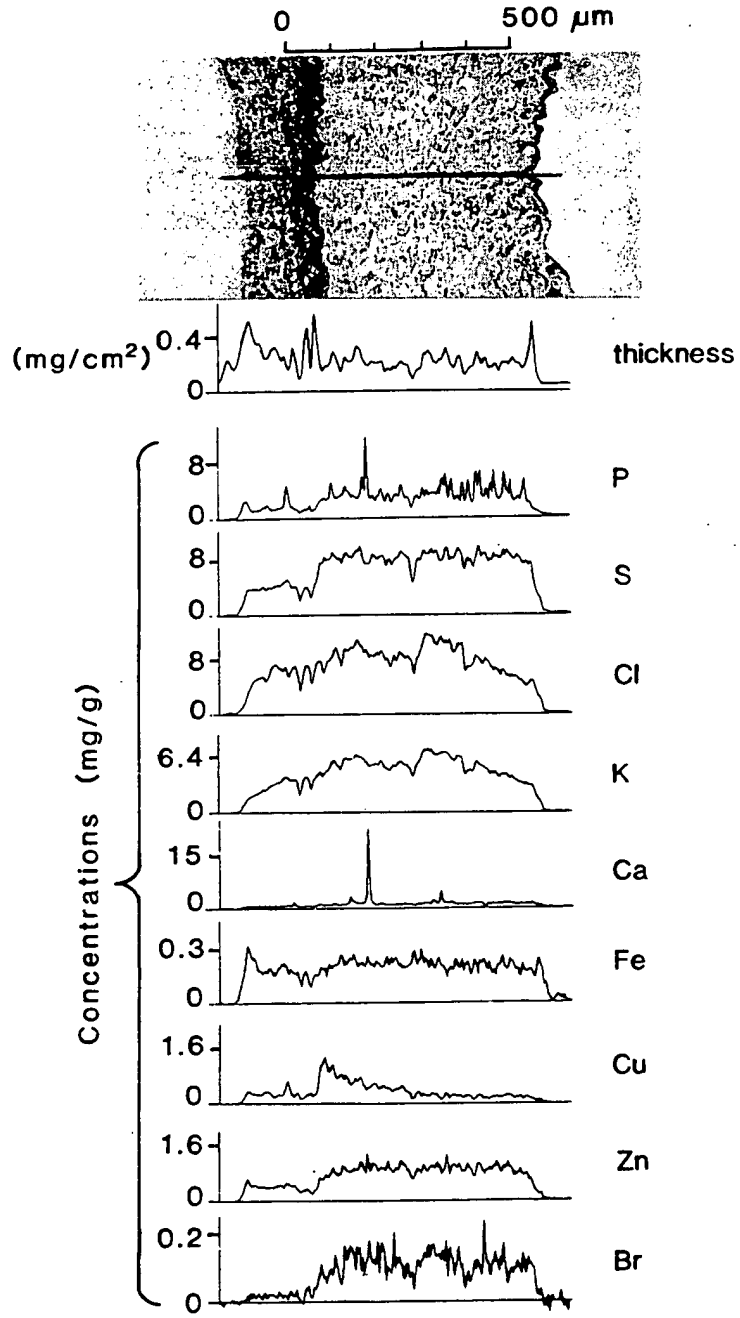

Fig. 1 — Human popliteal artery (x52). Unstained specimen from patient no. 1 showing the structure of the vessel wall. The sample thickness (mg/cm^2) and the distributions of element concentration (mg/g) along the scanning trace are given.

intima can be easily recognized. All elements show homogeneous distributions: only P and Ca distribution lines reveal places of focal accumulation, and distributions of these elements correlate. Both samples were obtained from young individuals whose artery walls were unaffected by atherosclerotic process. The highest concentration of Ca approaches 2% in sample no. 1 and 0.5% in sample no.

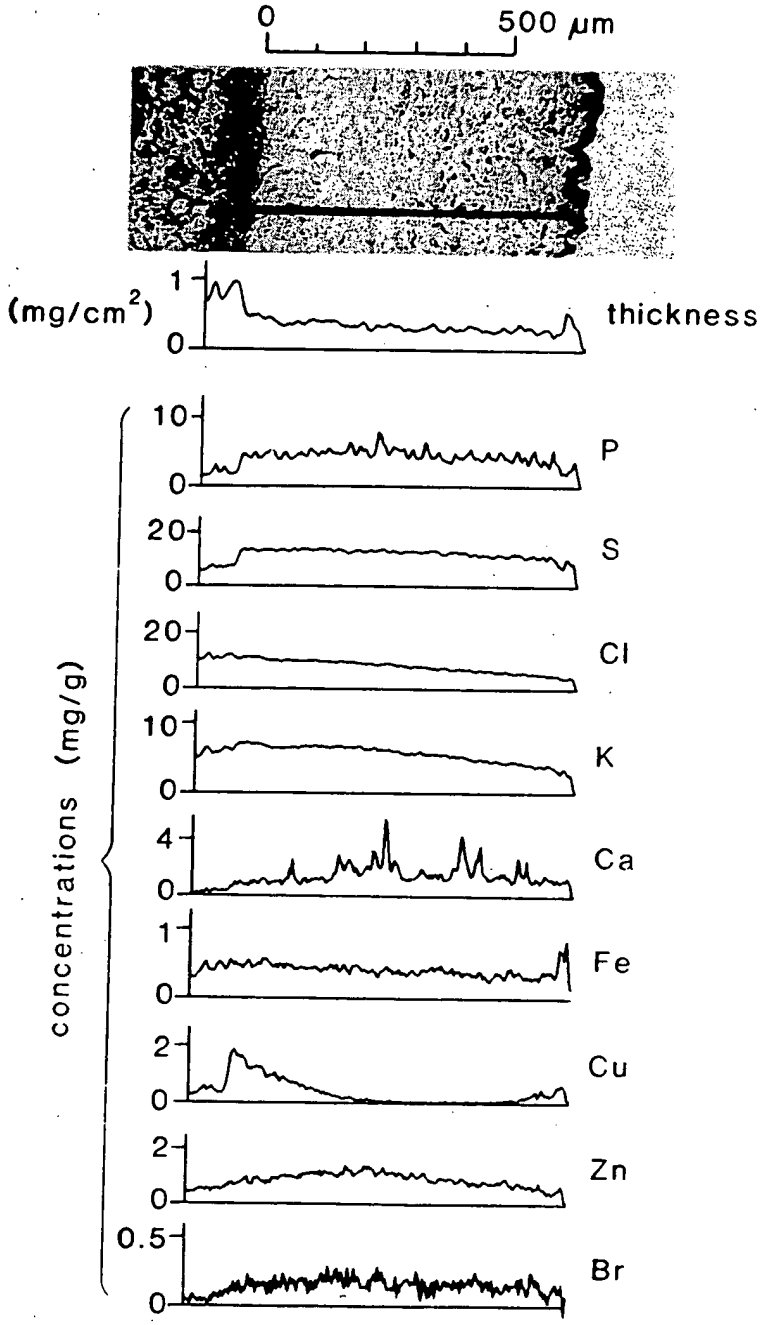

Fig. 2 — Human popliteal artery (x56). Unstained specimen from patient no. 2. For details see description of Fig. 1.

2. It is far below the level found in human arteries with advanced atherosclerotic lesions, where the maximum Ca concentration exceeds 20%. The maximum concentration of P approaches 1% in samples no. 1 and 2 and 9% in sample no. 13. The concentrations of P and Ca obtained in our study are in good agreement with observations of *Cichocki* et al. (14-15).

To distinguish between the various compounds (Table II) involved in the

TABLE II
List of Ca-P compounds which may constitute inorganic deposits in artery wall and values of the corresponding Ca/P weight ratios.

Formula	*Ca/P*
$CaHPO_4 \times 2H_2O$	1.29
$Ca_8H_2(PO_4)_6 \times 5H_2O$	1.72
$Ca_{10}(HPO_4)(PO_4)_6$	1.85
$Ca_3(PO_4)_2 \times 3H_2O$	1.94
$Ca_3(PO_4)_2$	1.94
$Ca_{10}(PO_4)_6 CO_3$	2.16
$Ca_{10}(PO_4)_6(OH)_2$	2.16

mineralization process, the Ca/P ratio has been calculated. On the basis of PIXE or micro-PIXE data only, one cannot distinguish between organic and inorganic phosphorus. Therefore, Ca/P ratios calculated for separate deposits as well as mean concentrations contain a systematic error introduced by an unknown content of organic phosphorus, lowering the ratios obtained in our study. The Ca/P ratios calculated for mean concentrations (Table I) do not exceed 1.14. When these ratios are reckoned for selected places containing deposits, the obtained values still differ in most cases from those characteristic for pure Ca-P compounds (Table II), however, we did not observe the higher value of the Ca/P ratio than characteristic for hydroxyapatite (HA). One can also postulate that the observed differences occur because we deal with the mixture of a few compounds. Probably, within the human artery wall less stable minerals may be formed first which later either are converted to HA or serve as nucleator for HA.

If we delimit our study to young individuals (Table I sample no. 1, 2 and 3), the plot of the P concentration against that of Ca (Fig. 3) shows that Ca/P ratio is in all cases but four lower than in brushite ($CaHPO_4$) $x2H_2O$, which possesses the lowest Ca/P ratio among inorganic Ca-P compounds detected in organic systems. Therefore the micro-PIXE results give only a qualitative proof of the presence of inorganic deposits in the popliteal artery wall of young patients. The precise characterization of the chemical form of the deposits is impossible. Simultaneously, we did not find any morphological changes in the artery wall. For that reason our results contradict the current concept which considers the onset of calcification as the process occuring only in advanced atherosclerotic lesions.

The examination of the IR spectra can throw more light on the problem of the growth of mineral deposits and their phase transformation. Fig. 4 shows the IR absorption spectra of 4 different incinerated popliteal artery samples (Table I no. 2, 6, 10 and 13) and synthetic HA in the region 2000-400 cm^{-1}.

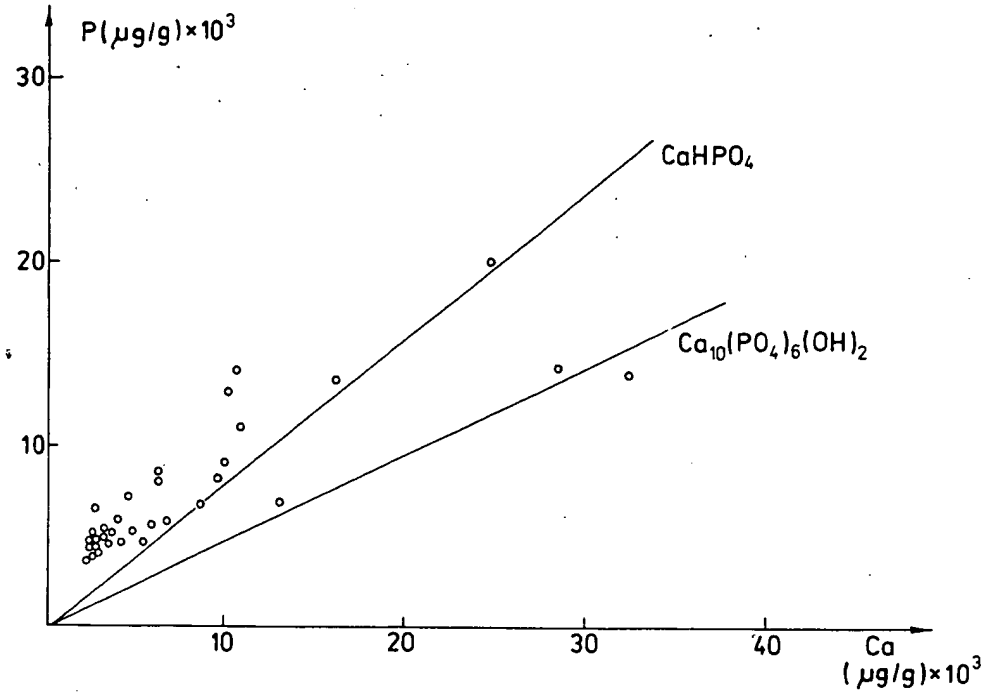

Fig. 3 — P concentration vs Ca concentration. The data were recorded in the mineral deposits within the popliteal artery wall. The concentrations are expressed in μg/g of dry mass.

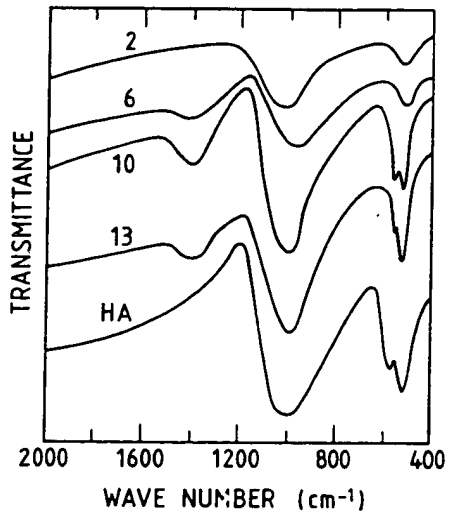

Fig. 4 — Typical infrared absorption spectra of samples no. 2, 6, 10 and 13 compared with that of hydroxyapatite (HA) in the region 2000-400 cm^{-1}.

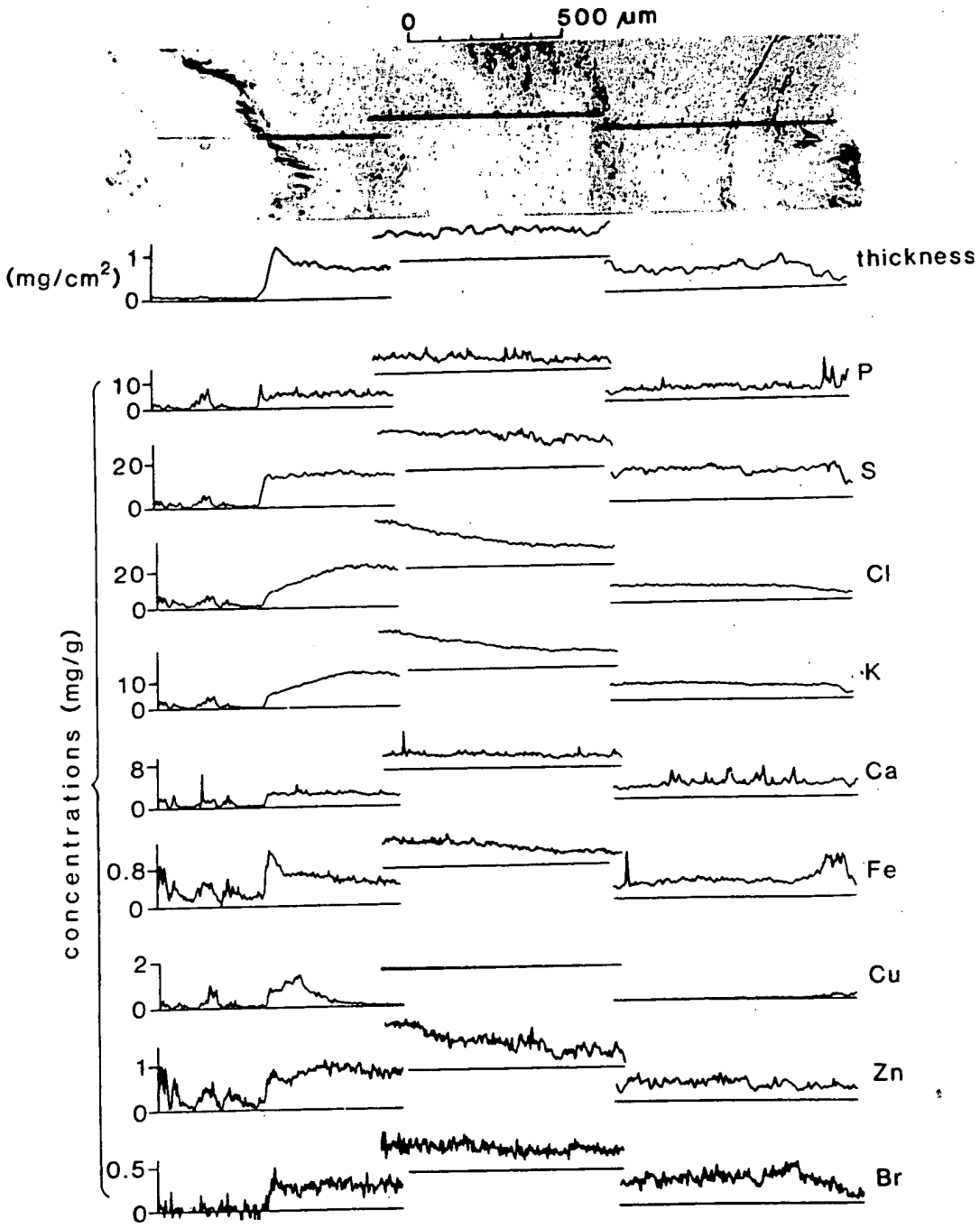

Fig. 5 — Human popliteal artery (x40). Unstained specimen from patient no. 3 showing the structure of the vessel wall. The sample was taken from the branching of the popliteal artery and the tibial artery. To cover the complete thickness of the wall three scans were performed. For details see description of Fig. 1.

The IR spectra of dried artery wall samples contain only bands due to organic molecules and are useless for the characterization of inorganic deposits. The bands due to PO_4^{3-} group at 1100-1000 cm^{-1}, 960 cm^{-1} and 600-550 cm^{-1} and CO_3^{2-} group at 1460-1410 cm^{-1} can be recognized in the spectra of the

ashed samples no. 10 and 13. The comparison of these spectra with the spectrum of HA, which was also incinerated for 8h at 450°C, gives the qualitative proof that the inorganic phase of mineralized artery wall is formed mainly by «apatitic» structures similar to that of HA. Moreover, the splitting of 600-550 cm^{-1} band from a broad singlet (sample no. 2 and 6), characteristic for amorphous calcium phosphate, to a doublet typical for the fully crystalline mineral (sample no. 10, 13 and HA) can be considered as indicative for crystallinity (16).

On the other hand, the bands due to CO_3^{2-} ions are distinctly present only in the samples no. 6, 10 and 13, i.e. samples taken from old patients. We are able to observe an interesting phenomenon: the content of Ca and P, the crystallinity of the mineral deposits as well as the amount of carbonate apatites (CA) correlate with the age of patients. On the basis of IR spectra we can conclude that there is a slow conversion of amorphous calcium phosphate to crystalline apatite, though it is not possible to recognize the intermediate phases. Moreover, in vitro precipitation of the solid phase from a carbonate-free medium does not lead to formation of CA (17-22). The presence of bands due to CO_3^{2-} ions in the spectra of samples taken from old patients indicate that the development of mineralization process is accompanied by an increased content of carbonate groups in the artery wall.

One can further speculate on the mechanism responsible for such changes. The increase in carbon dioxide content within the aging artery wall causes a pH decrease and creates conditions for CA formation. Lower pH may be also responsible for a precipitation of Ca-P compounds (Table II) other that HA (17), which are later transformed to HA. As a result, mineralized tissue should contain a broad spectrum of Ca-P compounds. This is in agreement with previously reported data (25).

The localization of Ca-P deposits within the artery wall is still an open question. We did not observe inorganic deposits within adventitia as well as at sites of vessel branching (Fig. 5). The latter observation is an additional proof of the lack of a relation between mineralization and the advancement of atherosclerotic changes; high shear stress at branching sites causes endothelial injury which is considered to be the first step in atherogenesis (26).

Some observations concerning the concentrations of elements other than Ca and P can also be made. Zn content at places of deposit locations is inversely proportional to Ca content, what confirms previously reported observations (14-15). Br and Zn concentrations are lowest in adventitia in all the investigated artery samples, probably reflecting the lowest cellularity of that layer.

CONCLUSIONS

The peaks in the distributions of P and Ca concentrations confirm the presence of Ca-P mineral deposits within the human popliteal artery wall. The minerals were found within tunica media. In the tunica adventitia as well as at sites of vessel branching the mineral deposits were not observed. No correlation was found between the presence of «small» mineral deposits (Ca concentration $< 1.5\%$) and morphological changes of the artery wall.

The amount of carbonate groups within popliteal artery wall as well as the crystallinity of the minerals correlate with the age of individuals. Probably, the increase in carbon dioxide content within the aging artery wall causes a decrease

in pH and creates the conditions for carbonate apatite formation. The lower pH may also be responsible for precipitation of Ca-P compounds other than hydroxyapatite.

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SUMMARY

Popliteal arteries from 14 individuals (17-85 y old) were investigated. The concentrations and localizations of P, S, Cl, K, Ca, Fe, Cu, Zn and Br were measured by means of PIXE and micro-PIXE methods. The presence of PO_4^{3-} and CO_3^{2-} groups was assessed using the IR technique. The amount of P and Ca increased with age approaching at places 9% and 20% and mineral deposits were detected in tunica media. At the same time an increase in the Ca/P ratio and in the crystallinity of deposits was observed. The samples from old individuals also contained more CO_3^{2-} groups. The concentrations and localization of Zn and Br showed artery wall layer-dependent changes. In some places of the artery wall, minerals were also found in young persons. They were not correlated with places of blood vessel branching.